A comparative study of *in vitro* chitin synthase activity in mucoraceous hosts of a mycoparasite

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Table of Contents

List of Tables

List of Figures

Abstract

A comparative study of *in vitro* chitin synthase activity in mucoraceous hosts of a mycoparasite:

Chitin synthase, the enzyme responsible for the synthesis of chitin in fungal cell wall was extracted from young hyphae of *Choanephora cucurbitarum* and *Phascolomyces articulosus*, susceptible and resistant hosts, respectively, to the mycoparasite, *Piptocephalis virginiana*. Crude enzyme was identified and characterized by measuring the incorporation of the substrate [^{14}C]-UDP-N-acetylglucosamine, into chitin. Most activity occurred in mixed membrane fraction. Inhibition of activity with Polyoxin D and activation with proteases, N-acetyl-glucosamine and magnesium and other ions was observed. Properties of the crude enzyme preparation such as cofactor requirement, V_{max}, apparent K_m value for UDP-GlcNAc, inhibition by Polyoxin D, response to pH and to temperature, and stability at 4°C were determined. Enzyme activity from both fungi displayed basically the same features as the corresponding enzymes reported from other mucoraceous fungi. However, the two preparations from *P. articulosus* and *C. cucurbitarum* differed from each other in their expressed activity (*i.e.*, the preparations from *P. articulosus* exhibited higher latency and higher specific chitin synthase activity than the corresponding preparations from *C. cucurbitarum*). Trypsin was effective in activation only over a narrow concentration range. Acid protease was the most effective activator. Endogenous protease estimation

indicated higher protease activity in C. cucurbitarum than in P. articulosus. The suggestion is made that regulation of chitin synthase activities may be related to host resistance in the mycoparasitic system.

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Thanks are also due to all my colleagues for their cooperation and moral support and making my stay at Brock an enjoyable one.

A special thanks to Janet Hastie for typing this manuscript.

Almas Begum.

Dedication

I wish to dedicate this manuscript to my parents, for their constant encouragement during the course of this study.

Introduction

Knowledge of host-parasite relationships between economic plants and microorganisms is fundamental to plant pathology, yet the process of obtaining the knowledge has been slow and difficult because of the complex nature of this relationship.

The parasitism of one fungus on another has been recognized by mycologists for nearly one hundred years. Interest in the physiological aspects of the host-parasite relationship, among fungi was stimulated by plant pathologists studying similar relationships between pathogenic microorganisms and their plant hosts. But the science of mycology was not ready for intensive study of mycoparasites until fungus physiology had yielded knowledge of culture techniques, nutritional requirements and basic physiological activities of a variety of fungi. With this knowledge, mycologists are now able to use mycoparasites as tools in studies of the basic principles of parasitism.

The choice of mycoparasite systems over systems of higher plants has certain advantages, such as saving of time and space. The environment can also be controlled rigidly and the host nutrition can be manipulated easily. The concepts of plant pathology such as resistance and susceptibility are wholly applicable to this system (Barnett and Binder, 1973).

In this laboratory, fungal species used are the members of the class zygomyccetes a class characterized by the presence of chitin and

chitosan as major cell wall components (Bartnicki-Garcia, 1968). Both Choanephora cucurbitarum (Berk and Rav.) Thaxter and Phascolomyces articulosus (Boedijn ex. Benny and Benjamin) of order Mucorales are hosts to a mycoparasite, Piptocephalis virginiana Leadbeater and Mercer, of order Zoophagales. This obligate, biotrophic, haustorial mycoparasite has a host range restricted strictly to members of the order Mucorales. All members are not equally susceptible to infection (Berry and Barnett, 1957), some are completely resistant, e.g., P. articulosus and others such as C. cucurbitarum, become resistant with age (Manocha and Golesorkhi, 1979).

Some progress has been made toward understanding the factors affecting parasitism of a few biotrophic mycoparasites. From the investigations of Armentrout and Wilson (1969), Manocha and Letourneau (1978) and Manocha and Golesorkhi (1979), it has been established that mycoparasitism of a susceptible host species involves contact of the parasite with the host species, penetration of the host, development of haustorium and finally establishment of nutritional relationship. These are essential steps for growth and development of the mycoparasite. In case of a resistant host species, penetration is often impaired and the death of the biotrophic parasite results.

Ultrastructure studies by Manocha (1981), have shown that resistance to parasitism in mycoparasite systems is physical and suggested that the contact between host and parasite invokes specific responses in the host at the penetration site. This results in the development of morphological structures such as papilla and sheath. The former blocks the penetrating hypha and the latter prevent the haustorium from establishing a direct

contact with the host protoplasm. Graham (1981), in an *in vivo* study using autoradiographic techniques, demonstrated that chitin synthesis was stimulated in the host species at the point of penetration by the parasite *Piptocephalis virginiana*, and resulted in the formation of the specific cell wall-like structures which surround the haustorium. The extent of stimulation of chitin synthase was found to be different in the resistant host, *P. articulosus* and in the susceptible host *C. cucurbitarum*. It has been suggested by Manocha (1981) that deposition of cell wall-like material may be involved in the mechanism of the host to resist the mycoparasite.

Based on the findings that an attempt by parasite to penetrate the resistant host probably stimulates a defensive reaction in the host by converting host enzyme from inactive to active form, with the appearance of morphological structures such as papilla or sheath around the invading haustorium, an *in vitro* comparative study of the properties of chitin synthase in the two host species, *C. cucurbitarum* (susceptible) and *P. articulosus* (resistant) has been undertaken, the aim of which was to find out if any differences exist in the regulation of the enzyme systems, which could account for *in vivo* observed differences in the two host species (Manocha and Graham, 1982).

Literature Review

This literature review has been organized into two sections with an intent to cover in detail the work and studies which have been done to date in the field of mycoparasitism. The first section deals with the role of host cell wall in host-parasite interaction. The second section is concerned with the synthesis of cell wall polymer, chitin, and the properties of the enzyme , chitin synthase.

I.

From the investigations carried out by Armentrout and Wilson (1969), Manocha and Lee (1971), Manocha and Letourneau (1978), Manocha and Golesorkhi (1979), it has been established that mycoparasitism of a susceptible host species involves contact of the haustorial parasite with the host, penetration of the host, development of haustorium and, finally, establishment of a nutritional relationship with the host. This relationship is an essential step for growth and development of the biotrophic mycoparasite. Differences in response to parasitic infection are believed to be related to the structure and/or composition of the host cell wall prior to the infection, as well as variations in the deposition of cell wall-like material induced during mycoparasitism (Manocha, 1981).

Chitin synthesis induction in the form of host specific structures in response to mycoparasitism and their possible role in the mechanism of resistance has been described by Graham (1981), in the host-parasite system consisting of Choanephora cucurbitarum, Phascolomyces articulosus and the biotrophic parasite Piptocephalis virginiana. The structures commonly observed are papilla and sheath; the latter surrounds the haustorium. These structures are known to be the product of localized chitin synthase activity in the resistant host. Graham (1981) showed that chitin synthesis is stimulated at the infection sites as shown by autoradiography. The extent of stimulation of chitin synthase was found to be different in resistant (P. articulosus) and susceptible (C. cucurbitarum) host species.

Ultrastructure studies by Manocha (1981) have shown that resistance to mycoparasite is physical and suggested that the contact between host and parasite invokes specific responses in the host at the penetration site. This results in the development of morphological structures such as papilla and sheath. Papilla blocks the penetrating hypha and the sheath prevents the haustorium from establishing a direct contact with the host protoplasm. Although formation of papilla is a frequent response of many plants to fungal infection, their role in the host-parasite relationship is not well understood (Aist, Kunog and Israel, 1979). The sheath, on the other hand, comprises the host-parasite interface, the region of direct interaction between the host and the parasite (Bracker and Littlefield, 1973). The sheath has been defined as the amorphous but

homogeneous matrix surrounding the invading parasitic hyphae, excluding the host membrane and parasitic cell wall (Littlefield and Bracker, 1972). Although these structures vary in size, shape and texture as a function of species, age and compatibility, each is known to be the product of localized activity of the host (Bracker and Littlefield, 1973).

Gradual development of the sheath with time has been reported in the electron microscopic studies of susceptible young hyphae of C. cucurbitarum infected with P. virginiana (Manocha and Lee, 1971; Manocha and Letourneau, 1978; Manocha and Golesorkhi, 1979). Resistant host species P. articulosus, however, exhibited a different response to infection by P. virginiana. Attempted penetration of the host wall has been reported to result in faster development of a small papilla, while continuous penetration resulted in the development of a sheath 18-24 hours following inoculation (Manocha and Graham, 1982). In resistant responses the sheath appeared to be a continuation of the inner layer of the host cell wall (Manocha and Golesorkhi, 1979). Autoradiographic investigations carried out by Manocha and Lee (1972) and Manocha and Letourneau (1978) showed that in cultures of young C. cucurbitarum exposed to tritiated N-acetyl-glucosamine at various times intervals after inoculation there was a direct correlation between the time of onset of sheath development and the extent to which the label was incorporated into the zone of infection. Up to 18 hours after inoculation, no label was observed at the host-parasite interface while extensive labelling was evident at hyphal apices. Labelling had been reported to increase slightly over the sheath at 28

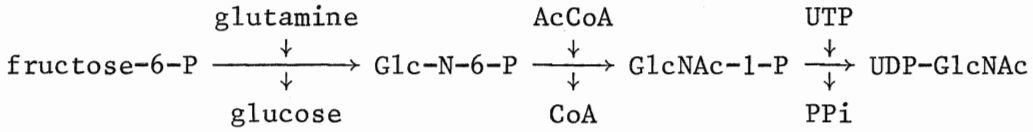
hours after inoculation. At 32 hours, grains were dense in the vicinity of the sheath. Microanalysis of [^{3}H]GlcNAc-labelled hyphae indicated that more than two thirds of the label was incorporated directly and specifically into cell wall chitin (Manocha and Letourneau, 1978). These data indicate that formation of the sheath is a gradual process probably induced by mycoparasitism.

The precise composition and origin of sheath is not known yet, due to the complexity and difficulty of its isolation from the mycoparasite system. However, indirect evidence involving the histochemical staining as well as incorporation of radioactive material has enabled speculation of concepts. Littlefield and Bracker (1972) suggested that the sheath might be composed of secretions either from host or parasite or both. In a later study, Bracker and Littlefield (1973) implied that the sheath acted as a buffer zone between host and parasite, and could therefore act as a binding site for materials from either partner. However, Mendgen and Heitefuss (1975), Sivak and Shaw (1970) indicated that no material is passed from parasite to host by autoradiographic studies of higher plants, nor does the sheath act as a sink for various substances released by the parasite (Mendgen and Heitefuss, 1975). Other autoradiographic investigations involving C. cucurbitarum infected with P. virginiana from this laboratory have illustrated that the sheath in this system is composed, at least in part, of material similar to that of the host cell wall (Manocha and Lee, 1972; Manocha and Letourneau, 1978) suggesting that this structure might be of host origin.

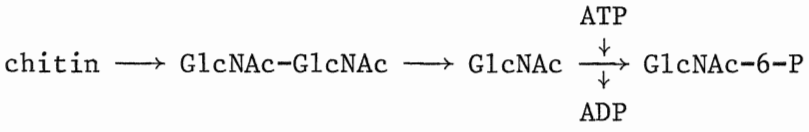
Like that of papilla, the exact role of the sheath in acquisition of resistant or susceptibility responses is not well understood. Based on information obtained from electron microscopic studies pertaining to the time of onset of development of structures such as the sheath, Manocha (1981) has proposed that rapid formation of papilla and sheath in resistant host species may be a mechanism of resistance to mycoparasitism. Rapid development of such material would prevent establishment of nutritional contact with host protoplasm and inhibit metabolic exchange of substances between parasite and host. Thus, failure of the biotrophic parasite to establish a nutritional relationship with a host species would result in its death. Since it is known that both the host cell wall and sheath are partially composed of chitin, it is possible that formation of the protective structure may be a result of localized chitin synthesis induced in response to mycoparasitic attack (Manocha, 1981).

II. Synthesis of cell wall polymer, chitin

Since chitin is a major component of fungal cell walls (Bartniki-Garcia, 1968), its synthetic pathway has been studied in great detail. Previous investigations indicate that the process of chitin synthesis is similar throughout the various taxonomic fungal groups (Gooday, 1978). Chitin formation is dependent on a supply of uridine diphosphate-N-acetyl glucosamine (UDP-GlcNAc). Four enzyme activities make up the biosynthetic pathway to this nucleotide sugar *de novo* from glutamine and fructose-6-phosphate.



An accessory pathway occurs when pre-existing chitin is hydrolyzed or when exogenous N-acetylglucosamine is supplied.



(Gooday, 1978)

The enzymes involved in these pathways have not been studied in as much detail as has chitin synthase itself.

The final step in chitin synthesis is the formation of (1-4)-β linkages between N-acetyl glucosamine residues. The polymerizing enzyme (uridin-5-diphosphate-2-acetamido-2-deoxy-D-glucose: chitin-4-β-acetamidodeoxyglucosyl transferase: EC2.4.1.16), usually named chitin synthase, was first described in Neurospora crassa by Glaser and Brown (1957), has since been detected in all groups of chitin-containing fungi (Jaworski et al., 1965; Camargo et al., 1967; Keller and Cabib, 1971; McMurrough et al., 1971; Jan, 1974; Gooday and de-Rousset-Hall, 1975; Moore and Peberdy, 1975) and its properties appear to be very similar from all of these sources. Eight enzymes have been implicated in the

biosynthesis of microfibrillar chitin from glucose (Chiew, Shepherd and Sullivan, 1980). The final enzyme in this pathway, catalyzing the polymerization of chitin from precursor subunits of N-acetyl-glucosamine (GlcNAc) is chitin synthase. Localization of chitin synthase and the site of synthesis of chitin is still controversial. In the mucorales, most of the chitin synthase activity was found associated with a heavy fraction containing cell walls (McMurrough et al., 1971; Peberdy and Moore, 1975). This has lead to the hypothesis that the enzyme is localized and functional in the wall (Bartnicki-Garcia, 1973). However, Jan (1974) proposed the plasma membrane as the principal location, the high activity in the wall fraction would then be due to contamination of this fraction with plasma membranes. In contrast, in Ascomycetes (Ryder and Peberdy, 1977; Lopez-Romero and Ruiz-Herrera, 1976) and Basidiomycetes (Gooday, 1977) most of the chitin synthase activity was recovered from a microsomal fraction and little activity was found in the wall fraction. Substantial evidence for the attachment of chitin synthase to the plasma membrane has been reported for yeast, Saccharomyces cerevisiae (Duran et al., 1975) and Candida albicans (Braun and Calderone, 1978). In addition, these workers found that most of the enzyme was present in the plasma membrane in an inactive or zymogenic state which could be activated by limited proteolytic digestion. Activation of chitin synthase by limited proteolytic digestion has since been found in many other fungi (Ruiz-Herrera and Bartnicki-Garcia, 1976; Van Laer and Carlier, 1978; Lopez-Romero and Ruiz-Herrera, 1976; Archer, 1977; Ryder and Peberdy, 1977).

Comparison of chitin synthase activity from various cell fractions (Jan, 1974; McMurrough and Bartnicki-Garcia, 1973; Peberdy and Moore, 1975; Ruiz-Herrera, Lopez-Romera and Bartnicki-Garcia, 1977; Vermerlen, Raeven and Wessels, 1979) shows that although chitin synthase activity is detectable in various locations within the fungal cell, it is predominantly associated with the plasma membrane and microsomal fractions. Purified membranes have been observed to have the greatest enzymic activity. Exposure of the outer membrane surface of intact cells of Phycomyces blakesleeanus to a labelled chitin precursor resulted in limited synthesis of chitin, while exposure of both surfaces using isolated cell walls with attached plasma membrane produced high concentrations of labelled chitin (Jan, 1974) have shown that the enzyme appears to be localized on or at least accessible from the cytoplasmic side of the plasmalemma. Furthermore, the addition of glutaraldehyde to the outside of isolated intact plasma membrane of Saccharomyces cerevisiae had little effect on chitin synthase activity, while similar addition to the inside of the membrane resulted in a 90% irreversible decrease in enzymic activity (Duran, Bowers and Cabib, 1975). These data indicate that chitin synthase is associated with the plasmalemma and is accessible primarily from the cytoplasmic side of the cell membrane. Further electron microscopy and high resolution autoradiography of regenerating protoplast of S. commune (van der Valk and Wessels, 1976, 1977) have shown that chitin is synthesized only in close association with the plasma membrane and provide evidence that chitin synthase is actually localized in the plasma membrane.

It has been implied (Duran and Cabib, 1978) that chitin synthesis must involve translocation of glucosamine units across the plasma membrane since the glycosyl donor, UDP-GlcNAc is an intracellular product. Yet the soluble cytoplasm has the enzymic capacity to synthesize chitin from UTP and phosphorylated N-acetylglucosamine (McMurrough et al., 1971) or from glucose (Chiew et al., 1980). Chitin microfibrils are laid down outside the plasmalemma, but neither the form nor the mechanism by which chitin is deposited outside the membrane is known.

Chitin synthase usually exists in a zymogenic state, but the cause of this latency has not been determined. Studies of in vitro chitin synthase have shown that activation may be obtained by certain proteolytic enzymes. Ruiz-Herrera and Bartnicki-Garcia (1976) implicated a similar means of activation for in vivo synthesis. The discovery by Cabib and Keller (1971) that mild proteolytic digestion of a chitin synthase preparation from yeast resulted in an increase of enzyme activity was later confirmed for chitin synthase from filamentous fungi and opened ways to speculate about the regulation of chitin synthase by proteases. Various models have been proposed to explain the regulation of chitin synthase activity in vivo and in vitro. Both for septum formation in yeast and apical growth in filamentous fungi, models were proposed involving proteases in the activation and inactivation of this enzyme. Ruiz-Herrera and Bartnicki-Garcia (1976) suggested a model of apical growth in Mucor rouxii whereby inactive enzyme deposited at the wall area of the apical

dome would be proteolytically activated, followed by a rapid inactivation by prolonged proteolysis during the transition from the tip wall to the lateral wall, resulting in a sharp gradient of wall synthesis. In contrast, results obtained with chitin synthase preparation from protoplast derived from apical and subapical regions of hyphae from Aspergillus fumigatus (Archer, 1977) and Aspergillus nidulans (Isaac et al., 1978) have been interpreted to indicate that apical regions contain chitin synthase in a largely activated state and that in subapical regions the enzyme is present as a zymogen. Cabib et al. (1973) demonstrated in Saccharomyces cerevisiae the separate locations of inactive chitin synthase and the vacuolar proteases and an activating factor. A third component, a heat-stable cytoplasmic proteinaceous inhibitor was also described by Cabib et al. (1974) in Saccharomyces cerevisiae and for Mucor rouxii (McMurrough and Bartnicki-Garcia, 1973). It is believed that the inhibitor inhibits the activating factor and may function as a regulatory factor to inactivate the vacuolar protease that spills into the cytoplasm, hence preventing random stimulation of the enzyme. The activation of chitin synthase zymogen by the action of proteases has been shown by endogenous proteases that bring about activation in vitro (Cabib et al., 1973; Ruiz-Herrera and Bartnicki-Garcia, 1976; Braun and Calderone, 1979; Campbell and Peberdy, 1979). Campbell and Peberdy (1979) isolated an endogenous neutral protease from Aspergillus nidulans which caused activation of chitin synthase zymogen 14 times greater than that obtained by trypsin treatment. It is possible, therefore, that endogenous proteases may play an important role in the regulation of chitin synthase in vivo.

Cabib (1975) and Cabib, Ulane and Bowers (1974) described how chitin synthase occurs in the cell membrane as a latent zymogen. These workers proposed a model of chitin synthase activation. The enzyme can be activated by the action of protease that is present in vesicles in the cytoplasm. The protease is specifically inhibited by a protein present in the cytosol. Thus the site and timing of initiation of chitin deposition may be controlled by the selective activation of zymogen molecules in the membrane by release of the protease from the vesicles at that point. The inhibitor will then ensure that the activation remains localized. A similar mode of activation has been implicated for *in vivo* synthesis (McMurrough and Bartnicki-Garcia, 1973; Ruiz-Herrera and Bartnicki-Garcia, 1976). An inhibitory component of chitin synthase was isolated in *Mortierella vinacea* (Peberdy and Moore, 1975) and *Mucor rouxii* (Lopez-Romero *et al.*, 1978) which unlike that from *Saccharomyces cerevisiae*, does not inhibit protease action on the zymogen but inhibits active chitin synthase directly. The role of the chitin synthase inhibitor in filamentous fungi is yet to be established, but Lopez-Romera *et al.* (1978) suggested that it is a component of the regulatory mechanisms of chitin synthesis. Gooday (1979) suggested regulation of chitin synthesis by factors such as Mg^{+2}, presence of the allosteric activator, N-acetyl glucosamine (as a product of chitinolysis) and regulation by the inhibitory reaction product, uridine diphosphate. Also the possibility was forwarded that the enzyme was not restrained by any regulatory process and that the amount of enzyme molecule might be the limiting factors for *in vivo* chitin synthesis.

Properties of Chitin Synthase

Polymerization of the acetylated monosaccharide N-acetyl glucosamine is known to be preceded by nucleosidal activation of the precursor subunit by uridine triphosphate (UTP) to form uridine-diphospho-N-acetyl glucosamine (UDP-GlcNAc), the substrate for chitin synthase. Chitin synthesis then occurs by the addition of the activated monomer to the chitin oligomer in the presense of chitin synthase as outlined in Equation 1.

$$\text{UDP-GlcNAc} + (\text{GlcNAc})_n \xrightarrow[Mg^{+2}]{\text{chitin synthase}} \text{UDP} + (\text{GlcNAc})_{n+1}$$

(Gooday, 1978)

Table A outlines the properties of chitin synthase obtained from various sources. In all cases examined so far, several divalent cations, particularly Mg^{+2}, stimulate the reaction strongly. Free N-acetyl glucosamine (GlcNAc) stimulates the reaction without being incorporated and probably acts as a positive allosteric activator (Camargo et al., 1967; McMurrough and Bartnicki-Garcia, 1973; de Rousset-Hall and Gooday, 1975). The presence of a primer does not seem necessary, as purified preparations of chitin synthase are very active without added primer (Gooday and de Rousset-Hall, 1975; Duran and Cabib, 1978). During polymerization, individual N-acetyl glucosamine (GlcNAc) residues are added to the non-reducing end of the growing chain in the β-1-4-configuration, characteristic of chitin (Gooday, 1977). Unlike other homopolymers such as glycogen, the addition of acetylated glucosamine subunits to the growing chitin chain

has been shown to occur without a primer, such as pre-existing chitin oligomers, still the mechanism by which the first two units become attached in this synthetic process is unknown (Duran et al., 1978).

The substrate uridine-diphosphate-N-acetyl glucosamine (UDP-GlcNAc) itself acts as an activator of the enzyme and plots of the reaction velocity against substrate concentration give sigmoidal curves (McMurrough and Bartnicki-Garcia, 1973; de Rousset-Hall and Gooday, 1975; Lopez-Romero and Ruiz-Herrera, 1976; Ryder and Peberdy, 1977; Ruiz-Herrera et al., 1977). Biochemical studies of Mucor rouxii (McMurrough et al., 1971) have indicated that chitin is the major polymer biosynthesized from uridin-diphosphate-N-acetyl glucosamine (UDP-GlcNAc) in the presence of chitin synthase. Analysis of product synthesized in an in vitro assay utilizing the cell wall fraction of Mucor rouxii produced chromatographically immobile substance insoluble in hot alkali characteristic of chitin. Hydrolytic enzyme was also tested, only chitinase caused substantial breakdown of the assay product from which chitobiose was recovered.

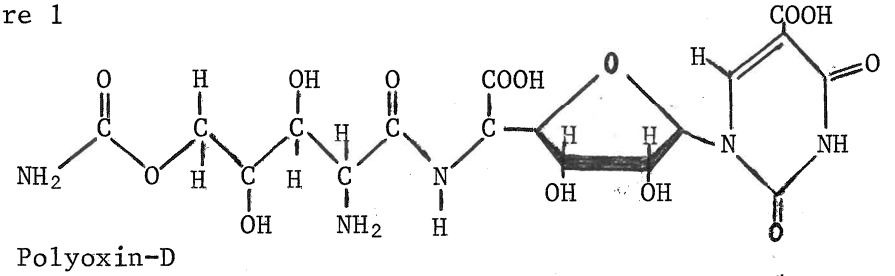
The enzyme product has been identified as macromolecular chitin by a variety of chemical and physical techniques. The most convincing evidence for its identity with the chitin of the cell wall comes from the demonstration that the enzyme product can be visualized as crystalline microfibrils (Ruiz-Herrera and Bartnicki-Garcia, 1974; Ruiz-Herrera, Sing, van der Woude and Bartnicki-Garcia, 1975). These authors solubilized the enzyme from a membrane fraction of M. rouxii by treatment at 0°C with

the substrate, uridine-diphosphate-N-acetyl-glucosamine (UDP-GlcNAc) and activator N-acetyl-glucosamine. Subsequent incubation gave a product giving a powder x-ray diagram characteristic of crystalline α-chitin. Electron microscopy showed single microfibrils emanating from granules, strongly suggesting a granular enzyme feeding out the macromolecular product (Gooday, 1971). Diacetyl chitobiose has also been reported as a minor product of the enzyme preparations from Blastocladiella emersonii and Mucor rouxii (Camargo, Dietrich, Sonneborn and Strominger, 1967; McMurrough and Bartnicki-Garcia, 1973). In the former case it was formed particularly at low concentrations of UDP-N-acetyl glucosamine, and in the latter case only in the presence of high N-acetyl glucosamine concentrations.

Chitin synthase isolated from various fungal species exhibits similarity in its response to different inhibitors. Polyoxins are a group of peptidyl pyrimidine nucleoside antibiotics (Fig. 1)

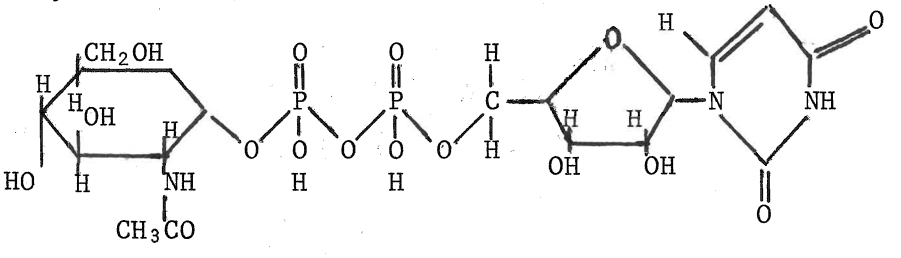
Figure 1

(a)



Polyoxin-D

(b)



UDP-N-acetyl glucosamine

The fungicidal activity of Polyoxin D on Neurospora crassa was investigated by Endo, Kakiki and Misato (1970). They found that treated hyphae had a greatly reduced incorporation of [^{14}C]-glucosamine into chitin. Inhibition by the antibiotic Polyoxin D, specific for chitin synthase has since been commonly observed in chitinous fungi. Competitive inhibition by this antifungal agent observed in kinetic studies of chitin synthase (Archer, 1977; Chiew et al., 1980; Ruiz-Herrera and Bartnicki-Garcia, 1976; Jan, 1974) has been attributed to its gross structural similarity to the substrate UDP-N-acetyl glucosamine. Although the effect of Polyoxin D on other enzymes utilizing this substrate has not been well documented, it is known that it is not a competitive inhibitor of UDP-GlcNAc in a pyrophosphorylase enzyme of N. crassa (Endo and Misato, 1969). Competitive inhibition by Polyoxin D is specific to chitin synthase as other cellular processes such as respiration, nucleic acid, protein and phospholipid synthesis are not altered by addition of this antibiotic (Endo, Kakiki and Misato, 1970). Not all fungi containing chitin are susceptible to polyoxins, when tested in vivo. Although Polyoxin D is known to be variably inhibitory towards chitinous fungi (Gooday, 1977), Candida albicans was reported to be unaffected by this antibiotic (Chiew et al., 1980). Uridine-diphosphate, a product of chitin synthase, is an inhibitor of its activity (Gooday, 1977) in Coprinus cinereus.

Materials and Methods

Materials and Methods

Organism and Culture: Cultures of Choanephora cucurbitarum (Berk and Ravi) Thaxter and Phascolomyces articulosus (Boedijn ex. Benny and Benjamin), were routinely maintained on solid medium consisting of malt-extract, 20 g; yeast extract, 5 g; and agar, 20 g; in one liter of distilled water at 23°C ± 1°C.

For extraction of enzyme, mycelia were grown from spores of either C. cucurbitarum or P. articulosus inoculated at a concentration of approximately 9 x 10^{10} mL^{-1} in a 500 mL Erlenmeyer flask containing 200 mL of liquid medium of the same composition as described above minus agar. The cultures were incubated for 24 h at 23°C ± 1°C on a gyratory shaker at 120 rev. min^{-1} (G-24 Incubator shaker, New Brunswick Scientific Co. Inc., Edison, New Jersey, U. S. A.).

Isolation of subcellular fractions from mycelial homogenates: Mixed membrane fraction (MMF) and cell wall fraction (CWF)

Subcellular fractions from mycelia homogenate were isolated by a method similar to that described by Ryder and Peberdy (1977). Mycelia were harvested by filtration through Whatman No. 4 filter paper using a Buchner funnel, and washed twice in cold distilled water and twice with cold 25 mM KH_2PO_4/NaOH buffer, pH 6.5. Subsequent steps were performed at 0-4°C. The washed mycelia were resuspended in the above buffer, mixed

with glass beads (0.45-0.50 mm diameter) and homogenized for 35 seconds at 4000 rev. min^{-1} in a Braun MSK Cell homogenizer cooled with compressed CO_2. Mycelia were centrifuged at 1000 x G for 5 min to obtain the cell wall fraction which was washed five times with buffer. The washings were added to the supernatant and centrifuged at 100,000 x G for 40 min using an IEC/B-60 Ultracentrifuge. The pellet (mixed membrane fraction, MMF) was resuspended in 25 mM KH_2PO_4/NaOH buffer pH 6.5, with the aid of a Potter Elvehjem homogenizer and used as the crude enzyme preparation. Preparations were used immediately.

Chitin synthase assay: Chitin synthase activity in cell wall and mixed membrane fractions of P. articulosus and C. cucurbitarum was measured according to the method described by Ruiz-Herrera and Bartnicki-Garcia (1974) with some modifications. Unless indicated otherwise, the standard chitin synthase reaction mixture contained 0.5 mM UDP[^{14}C]-GlcNAc (specific activity 0.2 µCi $µmol^{-1}$) from the Radiochemical Centre, Amersham; 20 mM GlcNAc; 0.2 mM ATP, 10 mM $MgCl_2$, 25 mM KH_2PO_4/NaOH buffer, pH 6.2, and enzyme in a final volume of 150 µL; where indicated the assay mixture also contained various amounts of different proteases or protease inhibitor. After incubation for 5 min at 25°C ± 1°C, the enzymic reaction was stopped by adding 25 µL of glacial acetic acid. The radioactivity incorporated from UDP[^{14}C]GlcNAc into the insoluble residue of the reaction mixture was washed with 20 mL of washing solution containing 1 M acetic acid and 95% ethanol (80:20 v/v) and filtered under vacuum on to a glass-fibre disc (Whatman GF/A, diam. 2.4 cm) held in a

Millipore filtration unit. The discs were dried at 70°C. Chitin formed during the reaction was retained on the filter discs, possibly bound to the precipitated protein (Ryder and Peberdy, 1977), and the soluble assay components were removed. The dried discs were placed in scintillation vials containing 15 mL of aqueous scintillation fluid [0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene and 2 g of 2,5-diphenyl-oxazole dissolved in 1 L of toluene]. Radioactivity was determined by scintillation counting for 10 min in a Searle Delta 300 liquid scintillation system. The quenching curve was determined by using an external standard. All assays were performed in duplicate. This assay method is rapid and reproducible, the difference between the duplicates being less than 5% of the mean value. Controls consisting of heated enzyme (in a boiling water bath for 2 min), were always run along with the standard enzyme preparations. Incubation mixture filtered through discs gave counts comparable to that of the background.

Chitin synthase activity was expressed as n moles of [^{14}C]GlcNAc incorporated from UDP-[^{14}C]GlcNAc into chitin per minute at 25°C. Specific activity was expressed as n mol min^{-1} mg^{-1} of protein in enzyme preparations. Protein was determined by Lowry's method using bovine serum albumin (Lowry et al., 1951).

Proteolytic activation of chitin synthase

To determine the optimal amounts of trypsin, acid protease, neutral protease for proteolytic activation, subcellular fractions were treated with various concentrations of proteases (Sigma Chemical Company, U. S. A.).

The crude enzyme preparation was preincubated with trypsin (crystalline, obtained from bovine pancreas) for 15 min at 25°C, after which the digestion was stopped by adding a double amount (by weight) of soybean trypsin inhibitor. In controls the trypsin inhibitor was added prior to trypsin. Enzyme preparations were also treated with acid protease (crystalline obtained from Aspergillus saitoi) and with neutral protease (crystalline obtained from Bacillus amyloliquefaciens) for 15 min at 25°C (Ruiz-Herrara et al., 1977).

Enzyme preparations activated with the above proteases were employed in chitin synthase assays as described earlier.

Characterization of the reaction product:

For characterization of product formed from UDP-GlcNAc by crude enzyme preparations from P. articulosus and C. cucurbitarum, a method similar to that described by Ryder and Peberdy (1977), with some modifications was followed. Ten reaction mixtures, each containing 0.5 nmol of UDP-GlcNAc and 20 mM of GlcNAc and 50 µL of mixed membrane fraction in a total volume of 150 µL were incubated for 1 h then inactivated with 25 µL of acetic acid. The mixtures were pooled and dialyzed against three changes of distilled water at 30 min intervals. The non-dialyzable material was centrifuged at 2000 x G for 10 min to yield a radioactive sediment and a clear supernatant. The radioactive sediment was washed twice with water and suspended in 5 mL of water. The suspension was stored at -20°C. Samples from the suspension were used for identification of biosynthesized product.

Identification of biosynthesized chitin:

A. Enzyme hydrolysis: A sample (0.8 mL) of the radioactive sediment described above was incubated with 5 mg of purified chitinase in 0.2 M sodium acetate (pH 5.0) for 8 h at 30°C. The hydrolysate was centrifuged at 2000 x G, for 10 min. The supernatant was subjected to descending chromatography on a Whatman no. 1 paper and developed in 95% ethanol/1 M ammonium acetate (7:3 v/v). Radioactive spots were detected by visualization, the amino sugars by spraying with 0.5% solution of ninhydrin in butanol, and N-acetylated amino sugars by spraying with p-dimethyl-aminobenzaldehyde. (Partridge, 1948). Spots were identified using glucosamine and N-acetyl-glucosamine standards. Chromatogram origins were excised and counted.

B. Acid hydrolysis: A sample (1 mL of suspension) was hydrolysed with 5 mL of 6 M HCl at 120°C under nitrogen for 4.5 h. The sample was dried in vacuo, the residue dissolved in distilled water was chromatographed by descending chromatography on Whatman No. 1 paper. The chromatogram was developed in 95% ethanol/1 M ammonium acetate (7:3 v/v). Radioactive spots were detected and identified as described above.

Estimation of endogenous proteases using two substrates:
(i) casein, (ii) Nα-benzoyl-DL-arginine-p-nitroanilide

(i) Measurement with casein as substrate:

Endogenous protease activity in mixed membrane fraction (MMF) and supernatant fraction of Phascolomyces articulosus and Choanephora cucurbitarum, was measured according to the method described by Rick Wirnt (1965). In this method, casein was hydrolyzed by enodgenous proteases with the formation of hydrolysis products, whose tyrosine and tryptophan contents were measured spectrophotometrically at 280 nm using a Bausch and Lomb Spectronic 20 after precipitation of the residual substrate. The standard reaction mixture contained 1 mL of casein substrate (5 mg mL^{-1}), 0.1 M phosphate buffer (pH 7.5) and sample 0.1 mL of MMF/supernatant. After incubation for 20 minutes at 35°C, the reaction was stopped with 3 mL of 5% trichloroacetic acid and allowed to stand for 30 min at room temperature and centrifuged for 20 minutes at 3000 x G. Blanks were run in which the reaction was stopped before the addition of enzyme. A standard curve using 1-5 μg mL^{-1} of trypsin was plotted.

The extinction of the experimental tube after subtraction of the blank extinction was used to calculate the enzyme activity. A unit of enzyme activity (TU^{cas}) is defined as the amount of trypsin which under defined conditions (20 minutes incubation at 35°C; final volume of the incubation mixture: 20 mL; after addition of trichloroacetic acid:

5 mL) liberates sufficient trichloroacetic acid soluble hydrolysis products so that the extinction at 280 nm increases by 1.00 in 1 minute. The specific activity unit was TU^{cas} mg^{-1} protein.

(ii) Estimation of protease activity using Nα-benzoyl-DL-arginine-p-nitroanilide

The method employed is similar to that described by Erlanger and Kokowsky (1961). A stock solution of Nα-benzoyl-DL-arginine (43.5 mg) was dissolved in 1 mL of dimethyl sulfoxide and the solution brought up to 100 mL with 0.05 M tris buffer (pH 8.2) containing 0.02 M $CaCl_2$. To 0.1 mL of enzyme solution was added 5 mL of substrate solution and the reaction allowed to run for 10 minutes in a thermostatically controlled bath at 25°C for 5 minutes. A suitable control without enzyme was also set up. The reaction was terminated by the addition of 1.0 mL of 30% acetic acid. The quantity of p-nitroaniline was estimated spectrophotometrically at 410 nm in a Bausch and Lomb Spectronic 20 using 19 x 150 mm cuvettes.

Results

Results

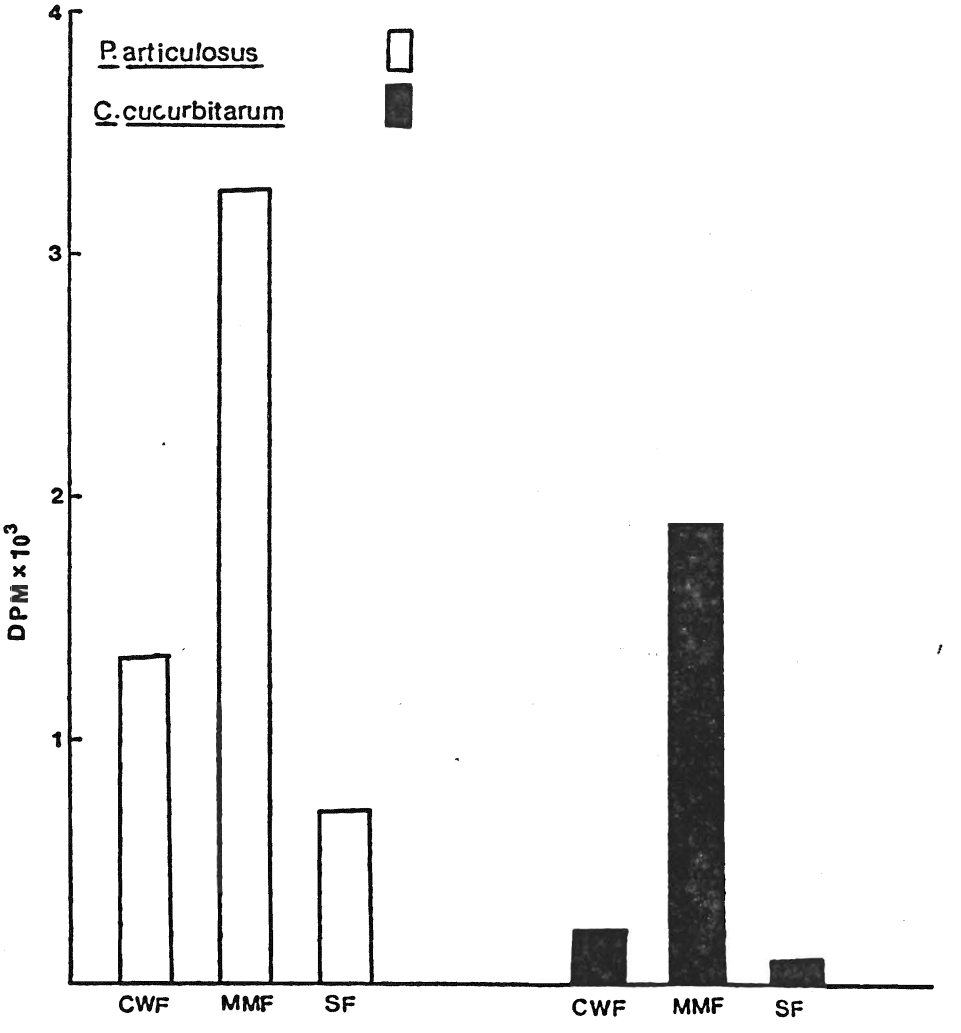
Chitin synthase activity in subcellular fractions of *Phascolomyces articulosus* and *Choanephora cucurbitarum*:

The chitin synthase activity in the various subcellular fractions obtained by ultracentrifugation was determined in the presence of acid protease at a final concentration of 200 μg mL^{-1} of enzyme preparation. The results are presented in Figure 1. For protease treatment, one mL of each fraction was preincubated with acid protease for 30 min at 25°C ± 1°C. Chitin synthase activity in the activated fraction was assayed as described in "Materials and Methods" using standard incubation mixture. Figure 1 shows bulk of the enzyme activity in the mixed membrane fraction (MMF) and the cell wall fraction (CWF), obtained from mycelia. Proteolytic treatment of the fractions revealed inactive enzyme in every fraction. The mixed membrane fraction contained 58-59% of the total enzyme activity. The low speed pellets (CWF) which contain high specific activity can be explained by the enrichment of the plasma membrane (MMF) fragments in this fraction. The data and Figure 1 show that the bulk of the enzyme activity was in mixed membrane fraction, hence this fraction was used in all subsequent experiments.

Figure 1. Chitin synthase activity in subcellular fractions from mycelium of Phascolomyces articulosus and Choanephora cucurbitarum:

Subcellular fractions from mycleium were prepared as described in "Materials and Methods". Chitin synthase activities after proteolytic activation were measured in various sedimental fractions. Each fraction was activated with 200 μg mL^{-1} of acid protease as described in "Methods". Samples of cell fractions (50 μL of enzyme preparation) were added to the standard reaction mixture in a final volume of 150 μL. Data are expressed as DPM x 10^3 incorporation of GlcNAc into chitin.

Figure 1


P. articulosus
C. cucurbitarum
DPM x 10³
4
3
2
1
CWF
MMF
SF
CWF
MMF
SF

Effect of exogenous proteases on chitin synthase activity:

Crude enzyme preparation was assayed for the expressed activity (i.e., the activity found immediately after cell disruption), and the total activity (i.e., protease activated activity). The data in Table 1 show the expressed activity and the total activity of chitin synthase in membrane preparations from Phascolomyces articulosus and Choanephora cucurbitarum, in the presence of three different proteases. Crude enzyme preparation was preincubated with three different proteases, an acid protease (crystalline, obtained from Aspergillus saitoi) a neutral protease (crystalline, obtained from Bacillus amyloliquefaciens) and trypsin (crystalline, obtained from bovine pancreas), at various concentrations as described in "Materials and Methods". Data in Table 1 and Figures 2 and 3 show the enzyme activity was markedly affected by the protease treatment. Acid protease was the most effective protease. The amount of chitin formed increased after incubation with low acid protease concentration reaching a maximum value at 150-200 $\mu g\ mL^{-1}$ of enzyme preparation. The results obtained with trypsin show an increase in chitin formation after incubation with low concentration, reaching a maximum value at 5-10 $\mu g\ mL^{-1}$ of enzyme preparation. At higher trypsin concentration, however, there was a decrease in enzyme activity. Results found with neutral protease for the enzyme preparation from P. articulosus show an increase in chitin synthase activity after incubation with low concentration, reaching maximum value at 5 $\mu g\ mL^{-1}$ of enzyme preparation. Enzyme activity was slightly inhibited at high neutral protease concentration. Enzyme preparation from C. cucurbitarum, treated with neutral protease shows no effect at low

Figure 2. Effect of acid protease on chitin synthase activity

Protease solution (freshly prepared in distilled water) was used to preincubate the mixed membrane fractions in the required concentrations, as indicated, for 30 min. Reactions were started by adding MMF suspended in 25 mM KH_2PO_4/NaOH buffer (pH 6.2). Incorporation of GlcNAc into chitin was measured as described in "Methods". Data expressed as nmoles of GlcNAc incorporated into chitin per min per mg of protein.

Figure 2

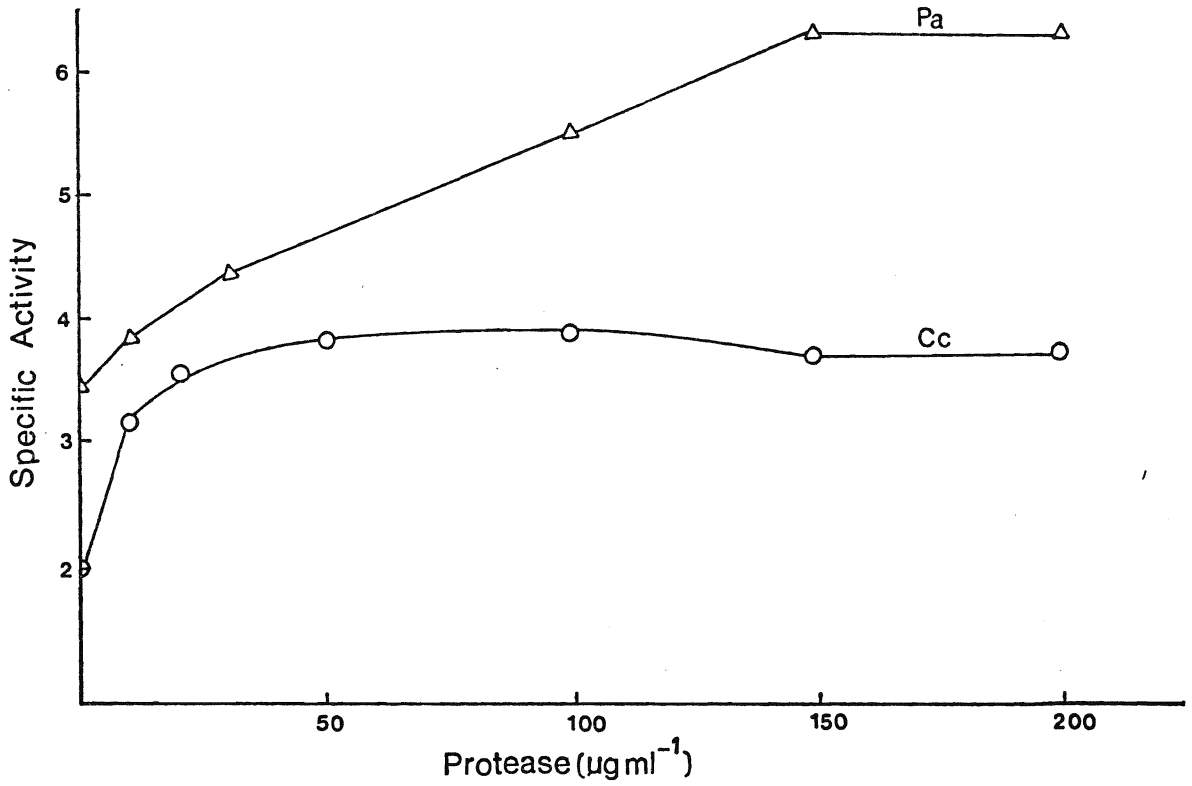


Figure 3. Effect of trypsin and neutral protease on chitin synthase activity:

Enzyme was prepared as described in "Methods". The mixed membrane fraction (MMF) was incubated for 15 min at 25°C ± 1°C with the indicated amounts of trypsin and neutral protease. Reaction was started by adding the protease treated enzyme fractions to the standard incubation mixture as described in "Methods". Data expressed as n moles of GlcNAc incorporated into chitin per min per mg of protein.

Figure 3

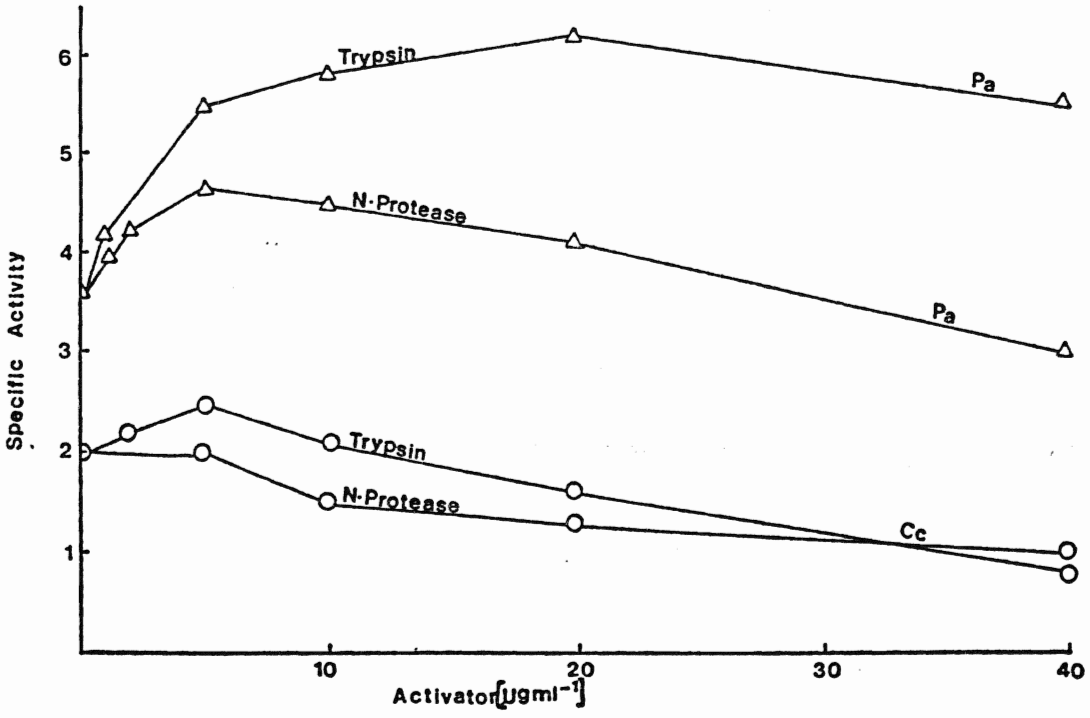

Specific Activity
6
5
4
3
2
1
Trypsin
Pa
N-Protease
Pa
Trypsin
N-Protease
Cc
10
20
30
40
Activator[μgml⁻¹]

Table 1. Effect of exogenous proteases on chitin synthase activity

	Phascolomyces articulosus			Choanephora cucurbitarum		
	Acid protease	Trypsin	Neutral protease	Acid protease	Trypsin	Neutral protease
Expressed specific activity (control without activitor)	3.6	3.6	3.6	2.1	2.1	2.1
Total specific activity (after treatment with proteases)	6.4	6.1	4.7	3.7	2.4	--
Activated activity (zymogenic form)	2.8	2.5	1.1	1.1	0.3	--

Mycelia were homogenized according to the procedure described under "crude enzyme preparations'. Homogenate was then fractioned through differential centrifugation, 100,000 G, 40 min. Pellet was resuspended in 25 mM KH_2PO_4/NaOH buffer pH 6.5 designated mixed membrane fraction. Chitin synthase activity was determined in MMF under standard conditions as described in method. The results show expressed specific activity (nmol GlcNAc incorporated min^{-1}(mg protein)$^{-1}$), without preincubating the enzyme preparation with protease. Total specific activity (after treatment with protease).

Table 4. Statistical analysis of inactive chitin synthase activity in the two host species, C. cucurbitarum and P. articulosus.

Acid protease	C. cucurbitarum	P. articulosus	t	p
	Inactive enzyme specific activity			
150 µg/mL	1.93	2.8	-4.24	<0.1>0.5
	1.47	3.2		
200 µg/mL	1.83	2.5	-3.7	<0.1>0.5
	1.47	2.3		
control	1.87	3.6		ns
	2.23	3.6		ns

ns = not significant

Table 5. Statistical analysis of total chitin synthase activity in the two host species, C. cucurbitarum and P. articulosus.

Acid Protease	C. cucurbitarum specific activity	P. articulosus specific activity	t	p
10 µg/mL	3.3	3.7	ns	ns
	3.5	4.0		(= P > 0.05)
20 µg/mL	3.7	4.1	ns	ns
	3.7	3.9		(= P > 0.05)
50 µg/mL	4.1	4.6	ns	ns
	3.8	4.5		(= P > 0.05)
100 µg/mL	4.2	5.2	-5.87	<0.05
	4.0	5.6		
150 µg/mL	3.8	6.4	-13.96	<0.01
	3.7	6.8		
200 µg/mL	3.7	6.2	-15.78	<0.01
	3.7	5.9		

ns = not significant

concentration, but at concentration above 5 µg of protease per mL of enzyme preparation, the enzyme activity was inhibited. The difference in response to inactive chitin synthase activity between C. cucurbitarum and P. articulosus at 150-200 µg/mL of acid protease fall just short at the required level of significance (t test, $0.1 > P > 0.05$; Table 4). There were no significant differences in the chitin synthase activity of two host species at acid protease treatment level from 10 to 50 µg/mL (t tests, $P > 0.05$). However, at higher acid protease treatment levels (100-200 µg/mL) there was a significant difference in the response of the two species (t tests, $P < 0.05$; Table 5).

Effect of Mg^{+2} and other cations on chitin synthase activity:

The enzyme activity increased with an increase in Mg^{+2} concentration. Figure 4 shows the requirement of the enzyme for magnesium ion (Mg^{+2}). The optimum concentration was 10 mM Mg^{+2} for both. Chitin synthase activity in the absence of metal ion was only 5-10% of the maximum activity. The effect of several other cations on chitin synthase was also investigated (Figure 5). Mg^{+2}, Mn^{+2} and Co^{+2} showed significant stimulation. These results are similar to those reported by Keller and Cabib (1971). Ca^{+2}, NH_4^+ and K^+ showed comparatively slight increase in activity. Zn^{+2} and Fe^{+3} caused the inhibition of chitin formation.

Effect of N-acetylglucosamine on chitin synthase activity:

The effect of different concentrations of N-acetylglucosamine (GlcNAc) on chitin synthase activity was investigated as indicated in

Figure 4. Effect of Mg^{+2} on chitin synthase activity:

A suspension of mixed membrane preparation in homogenizing buffer was activated with acid protease (200 μg mL^{-1}). Chitin synthase was assayed in this fraction using the standard incubation mixture containing varying amounts of magnesium chloride ($MgCl_2$) as indicated in Figure 4. Reaction was started by adding 50 μL of enzyme to 100 μL of reaction mixture. Data are expressed as n moles of GlcNAc incorporated into chitin per min per mg of protein.

Figure 5. Effect of metal cations on chitin synthase activity:

Assays were carried out using standard procedures described in "Materials and Methods", except for the cations used. The mixed membrane fractions (MMF) were used as the source of enzyme. The reaction mixture contained $MgCl_2$ and chlorides of other cations at a final concentrations of 10 mM. Enzyme preparations were activated by preincubation with acid protease (200 μg mL^{-1}) for 30 min.

Phascolymyces articulosus ■

Choanephora cucurbitarum □

Figure 5

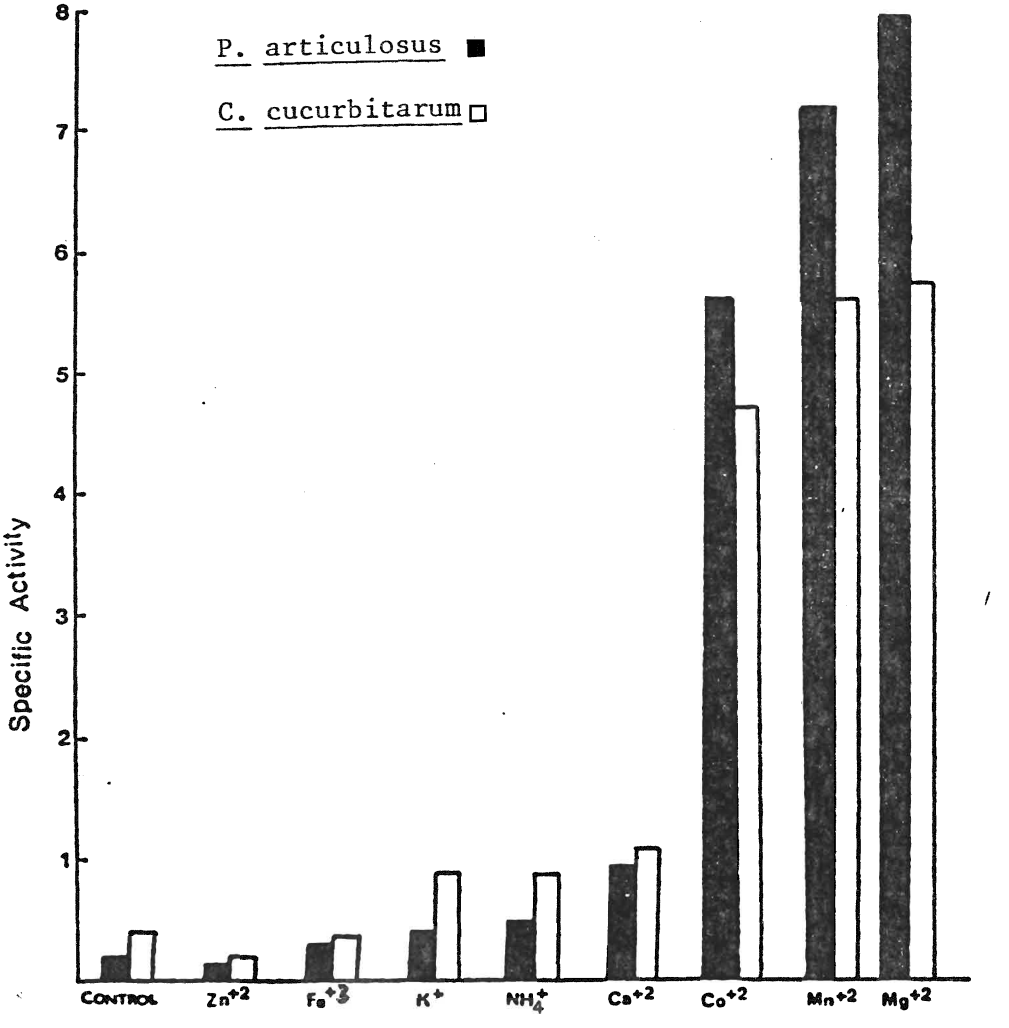

P. articulosus ■
C. cucurbitarum □
Specific Activity
8
7
6
5
4
3
2
1
CONTROL
Zn+2
Fe+3
K+
NH4+
Ca+2
Co+2
Mn+2
Mg+2

Figure 6. Effect of GlcNAc on chitin synthase activity:

Total chitin synthase activity was measured in each assay as described in "Materials and Methods", using varying amounts of GlcNAc. Enzyme preparation was activated with acid protease (200 μg mL^{-1}) for 30 min. Mixed membrane fractions (MMF) from Phascolomyces articulosus (Δ) and Choanephora cucurbitarum (○), were incubated with 100 μL of standard incubation mixture. Data are expressed as n moles of GlcNAc incorporated into chitin per min per mg of protein.

Figure 6

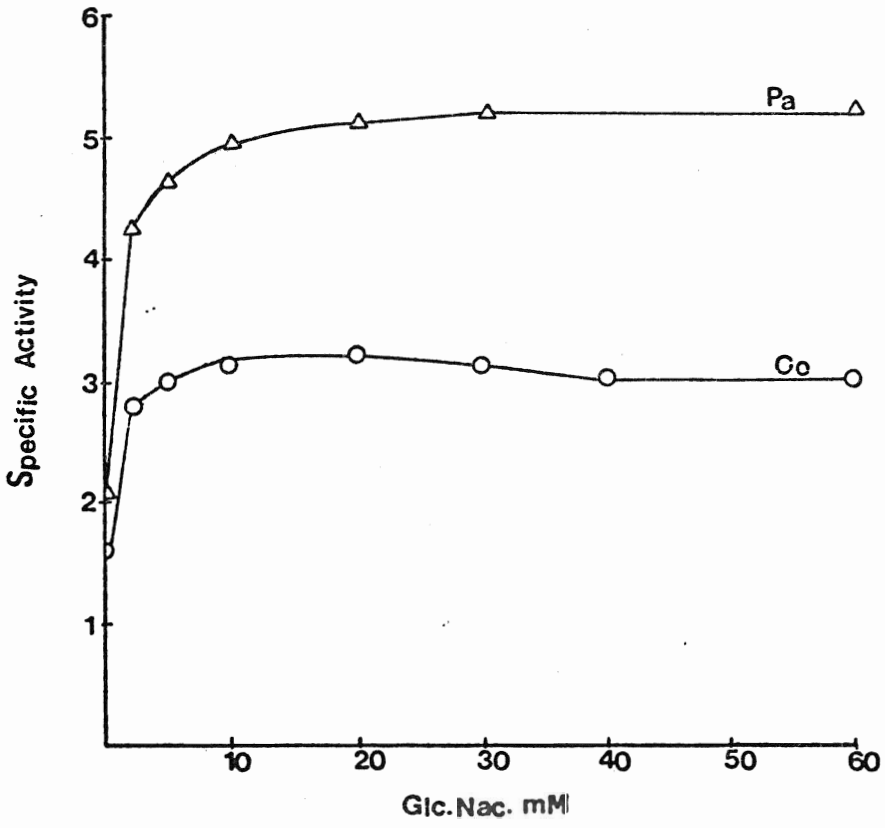


Figure 6. Chitin synthase activity in the absence of GlcNAc was 40-48% of the maximum activity in the two species. Figure 6 shows an increase in enzyme activity with the increase in GlcNAc concentration in the reaction mixture, which reaches a plateau when GlcNAc exceeds 30 mM.

Kinetics:

The amount of chitin synthesized in the reaction mixture increased linearly with time for at least the first 15 min (Fig. 9). It also varied linearly with the concentration of enzyme in the reaction mixture, in the range of 0.2-0.6 mg mL^{-1} of protein in the enzyme preparation (Fig. 10). The K_m value for the substrate uridine-diphosphate-N-acetylglucosamine (UDP-GlcNAc), was calculated by plotting Lineweaver-Burke plot. Assay conditions were the same except that various concentrations of substrate were used as indicated in Figures 7 and 8. The K_m value for UDP-GlcNAc, 1.7 mM and 2.5 mM, and V_{max} = 25 nmol and 50 nmol UDP-GlcNAc min^{-1} mg^{-1} of protein for C. cucurbitarum and P. articulosus, respectively. General properties of chitin synthase from P. articulosus and C. cucurbitarum are compared in Table 2.

Effect of Polyoxin D on chitin synthase activity:

The effect of Polyoxin D (5 µg mL^{-1} to 50 µg mL^{-1} of enzyme preparation) was studied at 0.5 mM UDP-GlcNAc concentration. Chitin synthase of P. articulosus and C. cucurbitarum were found to be strongly inhibited by Polyoxin D, as determined by the transfer of [^{14}C]-N-acetyl-glucosamine from [^{14}C]-UDP-N-acetylglucosamine to chitin. The inhibition

Figure 9. Time course for chitin synthase.

Assays were carried out using standard procedure described in "Materials and Methods". Standard incubation mixture was used as described in methods and incubated at different time intervals as indicated in Figure 9.

Choanephora cucurbitarum ○

Figure 9.

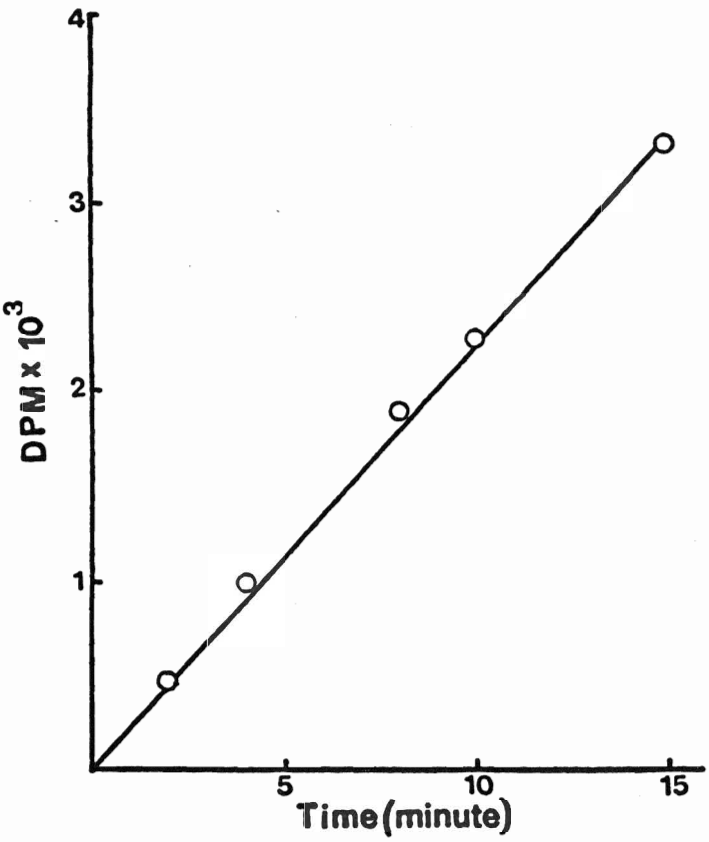
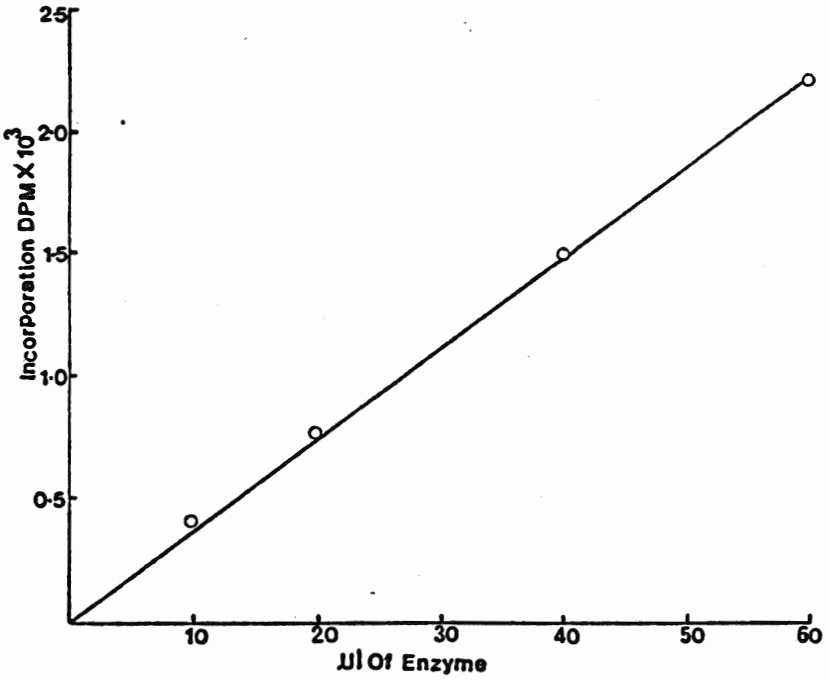


Figure 10. Variation of enzyme concentration and chitin synthase

Mixed membrane fraction was obtained as described in "Materials and Methods". The assay was done under standard conditions except for the variations in the amount of enzyme (MMF).

Choanephora cucurbitarum ○

Figure 10.


2·5
2·0
1·5
1·0
0·5
Incorporation DPM X 10³
10
20
30
40
50
60
µl Of Enzyme

Figures 7 and 8. Lineweaver-Burke plots of reaction rate dependence on UDP[^{14}C]-GlcNAc concentration.

Mixed membrane fractions (MMF) were obtained from Phascolomyces articulosus and Choanephora cucurbitarum, as described in "Materials and Methods". The assay was done under standard conditions, except for the variations in the amounts of uridine-diphosphate-N-acetylglucosamine (UDP[^{14}C]-GlcNAc) and Polyoxin D in the reaction mixture as indicated in Figures 7 and 8. Phascolomyces articulosus (Fig. 8): K_m = 2.5 mM; V_{max} = 50 nmole UDP-GlcNAc min^{-1} mg^{-1} of protein. Choanephora cucurbitarum (Fig. 7): K_m = 1.7 mM; V_{max} = 25 nmol UDP-GlcNac min^{-1} mg^{-1} of protein.

Choanephora cucurbitarum O

Phascolomyces articulosus △

Figure 7.

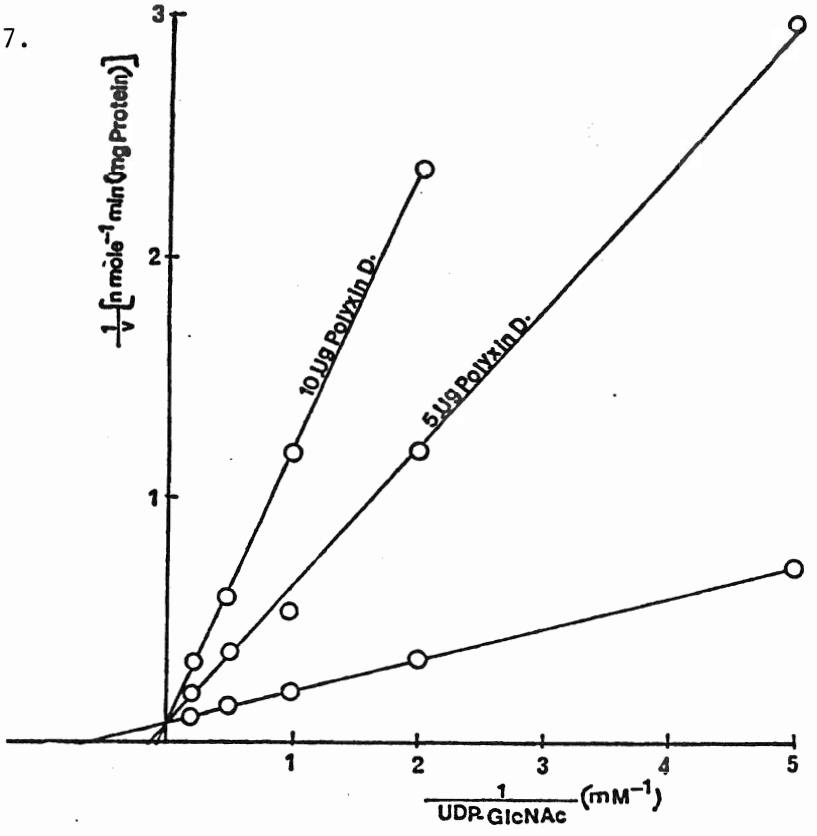


Figure 8

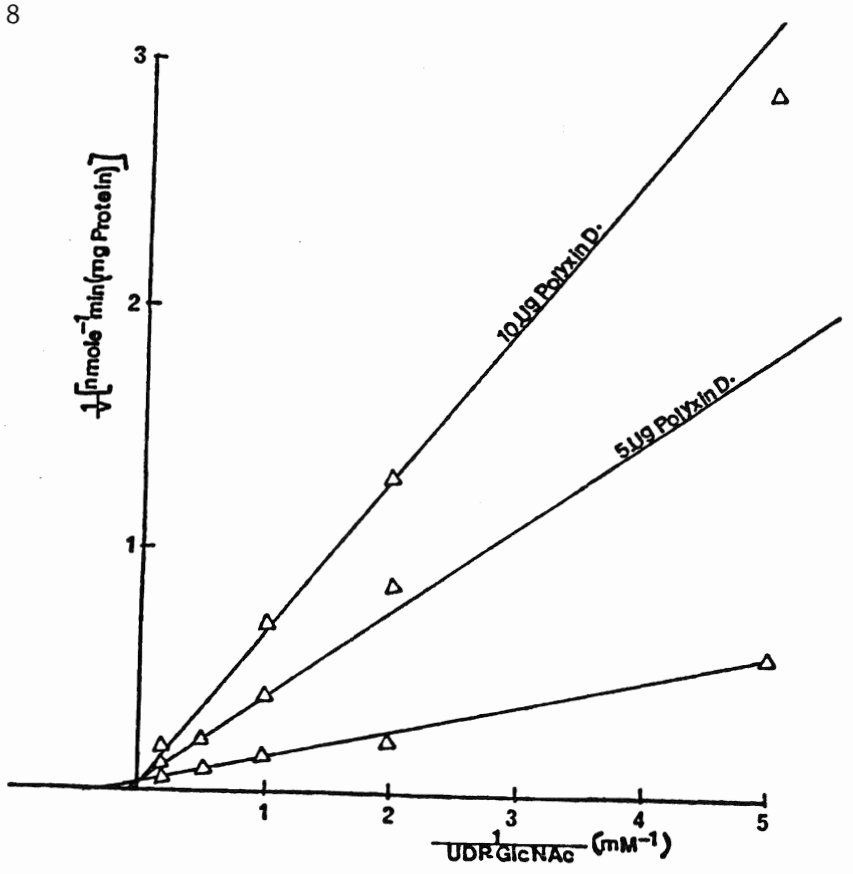


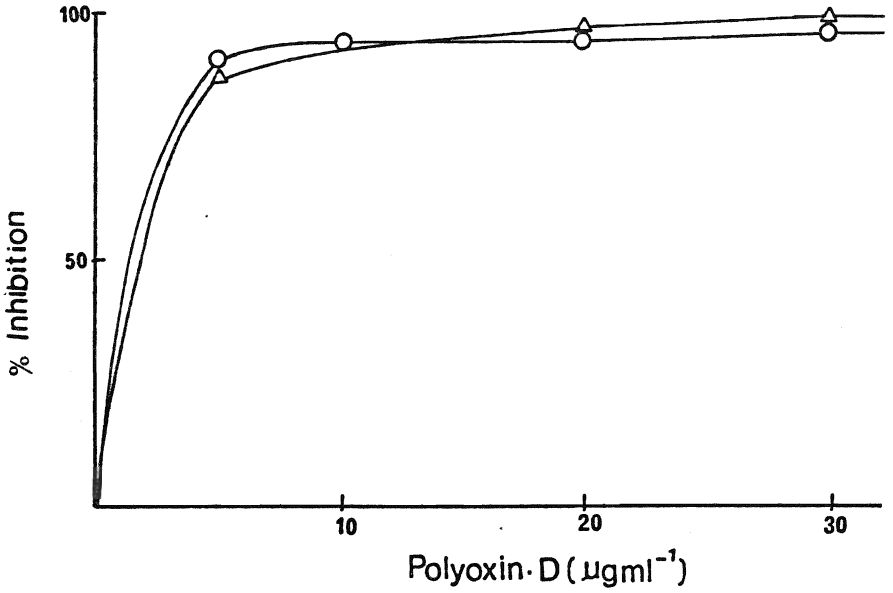
Figure 11. Effect of Polyoxin D on chitin synthase activity:

Conditions and concentrations of reactants were the same as indicated in "Materials and Methods" except the presence of varying Polyoxin D concentrations as indicated in Figure 9. The assay was done under standard conditions. Data are expressed as nmoles of GlcNAc incorporated into chitin per min per mg of protein.

Plascolomyces articulosis: △

Choanephora cucurbitarum ○

Figure 11.



was found to be competitive with respect to the substrate uridine-diphosphate-N-acetylglucosamine (UDP-GlcNAc). Figures 7 and 8 show competitive inhibition of chitin synthase by Polyoxin D. Date were plotted according to Lineweaver-Burke plot. Figure 11 shows the percent inhibition of chitin synthase at various concentrations of Polyoxin D as indicated in Figure 11. It shows the enzyme activity was markedly affected. 89-90% inhibition at concentration 5 $\mu g\ mL^{-1}$ and 96-97.5% at 50 $\mu g\ mL^{-1}$ concentration of Polyoxin D was obtained.

Effect of storage on chitin synthase activity:

Mixed membrane fractions (MMF) from Phascolomyces articulosus and Choanephora cucurbitarum were obtained as described in "Materials and Methods". Enzyme activity on storage over 48 hours at 4°C is shown in Figure 12. In the case of P. articulosus, activated and non-activated enzyme shows a gradual decrease in enzyme activity with time. However, enzyme preparations from C. cucurbitarum show rapid decrease within the first 5 h and then a sharp decline in activity thereafter.

Endogenous proteases:

Endogenous protease activity showed substrate specificity. Using casein as substrate, 25 $TU^{cas}\ \mu g^{-1}$ of trypsin and 6 $TU^{cas}\ \mu g^{-1}$ of trypsin, was obtained for mixed membrane fraction (MMF) and supernatant fractions of Choanephora cucurbitarum, respectively. 20 $TU^{cas}\ \mu g^{-1}$ of trypsin and 5 $TU^{cas}\ \mu g^{-1}$ of trypsin was obtained for the same fractions

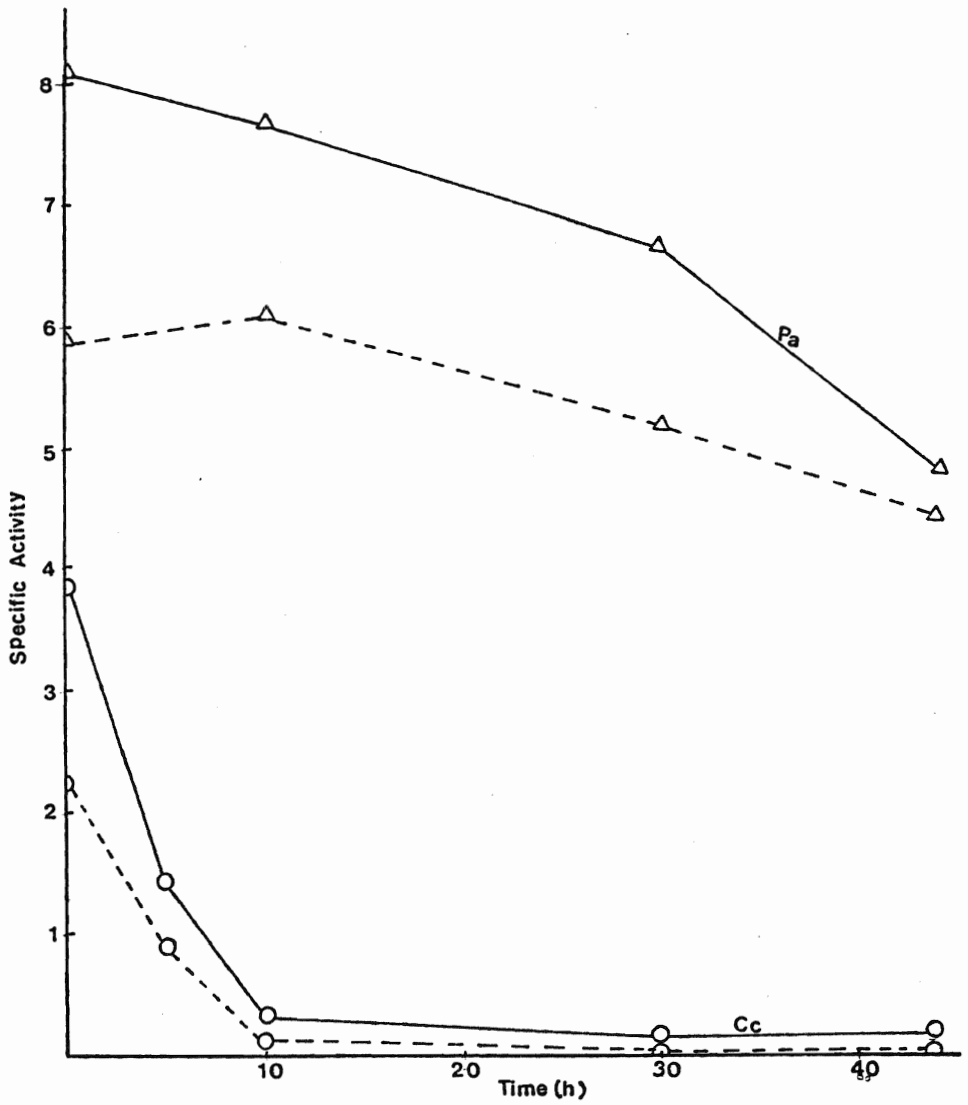
Figure 12. Effect of storage on chitin synthase activity:

Mixed membrane fraction (MMF) from Phascolomyces articulosus and Choanephora cucurbitarum were obtained as described in "Methods". The enzyme was stored at 4°C. The same standard reaction mixture was used throughout the experiment. Assays were performed at different time intervals as indicated in figure, in both activated (——) and non-activated (---) enzyme preparations. Enzyme was activated with acid protease (200 μg mL^{-1}) for 30 min at 25°C. Data are expressed as nmoles of GlcNAc incorporated into chitin per min per mg of protein.

Choanephora cucurbitarum ○

Phascolomyces articulosus △

Figure 12.



of Phascolomyces articulosus. There was no measurable protease activity using synthetic substrate (Nα-benzoyl-DL-arginine-p-nitroanilide). Results are summarized in Table 3.

Effect of pH on chitin synthase activity:

The effect of pH was tested on chitin synthase activity (MMF) from P. articulosus and C. cucurbitarum. The pH of the reaction mixture was varied using 25 mM (KH_2PO_4/NaOH) phosphate buffer. As shown in Figure 13, the synthesis of chitin had an optimum at pH 6.2. At values below pH 6.0, there was low chitin synthase activity in both host species.

Identification of reaction product:

Chromatograms of reaction mixture showed only two radioactive spots, one at the origin and one corresponding to uridine-diphosphate-N-acetyl-glucosamine (UDP-GlcNAc) standard, indicating the absence of glycosyl products. Chitinase treatment solubilized 95% of the reaction product giving a single radioactive product identified as GlcNAc. The reaction product was insoluble in 1 M acetic acid, but was completely hydrolyzed by 6 M HCl. Products of hydrolysis were identified as glucosamine. HCl hydrolysis yield was 77% of radioactive soluble fraction and chitinase digestion produced 95% of soluble radioactivity. The acid soluble radioactivity was identified as glucosamine by paper chromatography and of enzyme digested radioactive product was mainly identified as N-acetylglucosamine (GlcNAc).

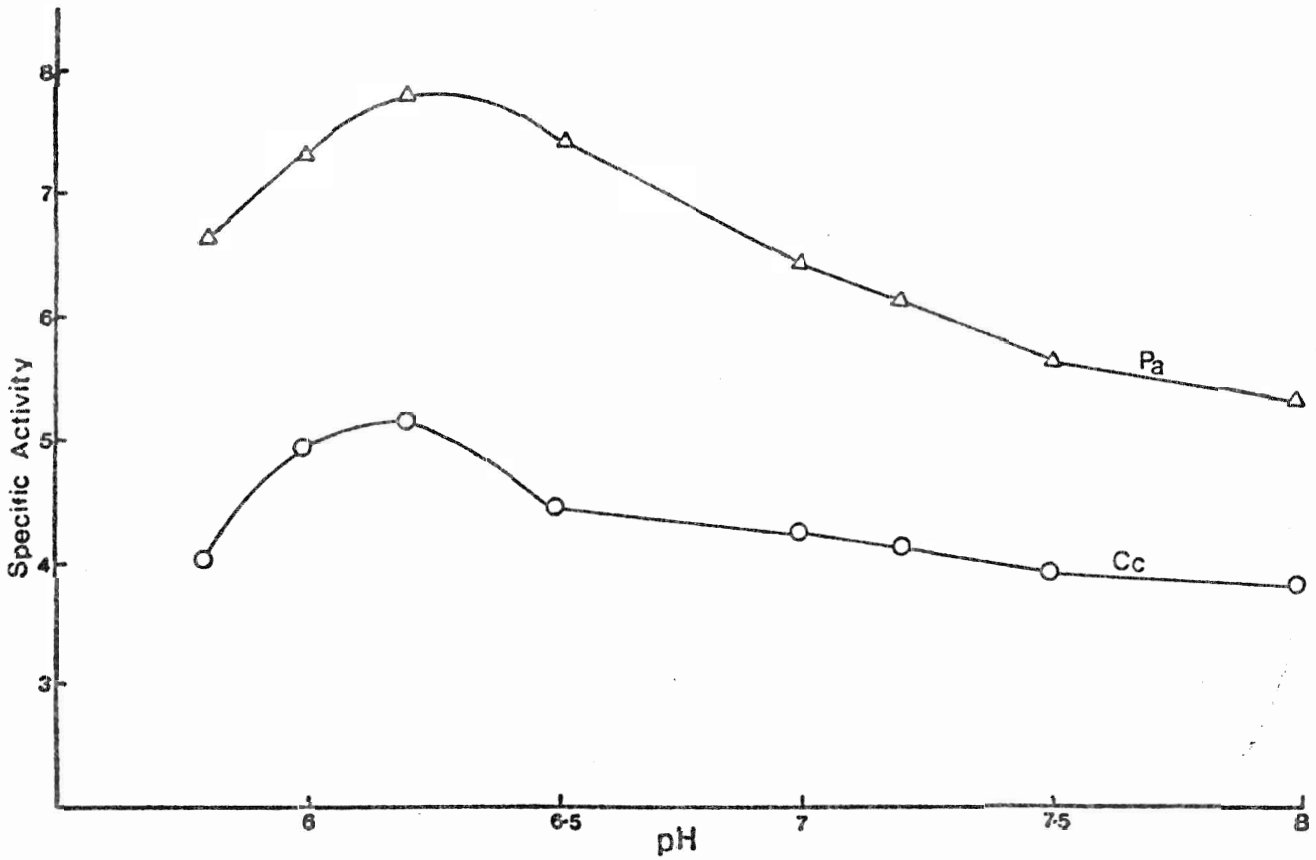
Figure 13. Effect of pH on chitin synthase activity:

Condtions for assay were the same as indicated in "Materials and Methods", except that pH of reaction mixture was varied using phosphate buffer at a final concentration of 25 mM.

Phascolomyces articulosus △

Choanephora cucurbitarum ○

Figure 13.



Discussion

Chitin synthase activity was measured in this study by a method similar to that described by Ruiz-Herrera and Bartnicki-Garcia (1976), as described in "Methods". This procedure yields results comparable to that obtained by chromatography of the reaction mixture, a method described by Peberdy and Moore (1975). The former has the advantage of being rapid and readily reproducible. The difference between the duplicates was less than 5% of the mean value.

Results from this study indicate that crude preparations of chitin synthase from P. articulosus and C. cucurbitarum, resembled the enzyme obtained from other fungi in its general requirements. Chitin synthase from the two species was identified and characterized by measuring the incorporation of the substrate [^{14}C]-UDP-GlcNAc into chitin. The reaction product was identified as macromolecular chitin by a variety of chemical and physical techniques. Paper chromatography of HCl hydrolyzed product revealed that about 80% of soluble radioactivity was GlcNAc. The most convincing evidence for the identification of in vitro synthesized chitin with the chitin of the cell wall comes from the demonstration that the enzyme product can be visualized by electron microscopy as crystalline microfibrils (Ruiz-Herrera and Bartnicki-Garcia, 1974; Ruiz-Herrera et al., 1975).

Subcellular distribution of chitin synthase in mycelium disrupted with MSK Braun cell homogenizer, that permits gentle lysis of cells and isolation of their plasma membranes, indicates that the bulk of the activity was located in the 100,000 x G fraction (Fig. 1). This constitutes about 58-59% of the total activity. Most of the remaining activity was associated with the 1000 x G fraction (CWF). Jan (1974) separated the membrane fraction from Phycomyces blakesleeanus, by centrifugation in a sucrose density gradient and found that chitin synthase activity correlated well with the activity of 5'-nucleotidase, which is considered a marker enzyme for the cell membrane in eukaryotes. Purified cell membranes from cells of Saccharomyces cerevisiae, prepared by Duran, Bowers and Cabib (1975), contained nearly all of the recoverable chitin synthase activity. Comparison of chitin synthase activity from various cell fractions (Jan, 1974; McMurrough and Bartnicki-Garcia, 1973; Peberdy and Moore, 1975; Vermerlen, Raeven and Wessels, 1979) suggests that although chitin synthase activity is detectable in various locations within the fungal cell, it is predominantly associated with the plasma membranes. The observations by McMurrough et al. (1971) that the enzyme is located on the cell wall could be due to contamination of the cell wall with membranous fractions.

Properties of the crude enzyme preparation, such as cofactor requirement, V_{max}, and apparent K_m value for UDP-GlcNAc were determined. Values obtained are comparable to those of other fungi (Table A, Appendix).

Magnesium ion (Mg^{+2}) and N-acetylglucosamine (GlcNAc) greatly stimulate the enzyme activity (Cabib and Bowers, 1973). The optimum concentration for Mg^{+2} was 10 mM and the activity in the absence of metal ion was only 5-10% of the maximum activity. This is comparable to Mg^{+2} requirements reported for other fungi. Experiments in which $MgCl_2$ was replaced by other chlorides showed that other divalent cations could replace Mg^{+2}. Mn^{+2} and Co^{+2} showed marked stimulation of enzyme activity comparable to that of Mg^{+2}. These results are similar to those reported by Keller and Cabib (1971). Ca^{+2}, NH_4^+ and K^+ showed comparatively slight stimulation of activity. Zn^{+2} and Fe^{+3} were inhibitory. The exact role of divalent cations is not known. They might, however, play a role in regulating the enzyme rate of reaction by binding at a site on the enzyme. This possibly will have to be confirmed in a purified enzyme system. As reported for other fungi, chitin synthase activity in the two species was strongly stimulated by N-acetylglucosamine (GlcNAc). It has been suggested that GlcNAc might act as a positive allosteric effector of the enzyme (McMurrough and Bartnicki-Garcia, 1973; de Rousset-Hall and Gooday, 1975).

In A. nidulans the effect of adding GlcNAc was found to be similar to that of high substrate concentration of activity. Irrespective of the molecular mechanism, the stimulation of chitin synthase by substrate (UDP-GlcNAc) and effectors like GlcNAc and Mg^{+2} is clearly an important potential regulatory system for the fine control of chitin synthase in vivo (Ryder and Peberdy, 1977). It has been further suggested that GlcNAc

resulting from chitinase action on chitin might locally stimulate chitin synthase activity *in vivo*. Degradation of chitin would thus stimulate its synthesis a coupling that may be important in wall extension at the hyphal apex. According to Gooday (1971), the allosteric activator role of GlcNAc should be considered an observation of purely laboratory significance, as this sugar apparently does not occur in measurable quantities in fungal cells. The only place that it will occur is at the site of enzymic lysis of chitin, by a concerted action of chitinase and diacetylchitobiase. It has been further suggested that the enzyme is not restrained by any regulatory process and that the amount of enzyme molecule might be the limiting factor for *in vivo* chitin synthesis (Gooday, 1979).

Accurate understanding of the regulation of chitin synthase requires a detailed knowledge of many parameters as discussed earlier, such as activators, cofactors requirement *etc*. Knowledge of inhibitors is also one of the important approaches towards the understanding of enzyme regulation. Chitin synthase isolated from *P. articulosus* and *C. cucurbitarum*, exhibited, 89-90% inhibition at 10 $\mu g/mL^{-1}$ of Polyoxin D concentration (Fig. 9), in the reaction mixture. The two fungal species exhibit similarity in their response to Polyoxin D. Competitive inhibition by this antifungal agent observed in kinetic studies of chitin synthase from many fungal species (Archer, 1977; Chiew *et al.*, 1980; Ruiz-Herrera *et al.*, 1976), is due to its gross structural similarity to the substrate UDP-GlcNAc. Competitive inhibition by Polyoxin D has been observed in chitin synthase activity of *P. articulosus* and *C. cucurbitarum* (Figs.

7 and 8). Inhibition by Polyoxin D is specific to chitin synthase as other cellular processes such as respiration, nucleic acid, proteins and phospholipid synthesis are not altered by the addition of this antibiotic (Endo, Kakiki and Misato, 1970).

Chitin synthase is synthesized as the proenzyme (Cabib and Farkas, 1971). The actual cause of this latency has not been determined. Activation of chitin synthase *in vitro* by proteolytic enzymes has been found in enzyme preparations from many fungi (Cabib and Ulane, 1973; Ruiz-Herrera *et al.*, 1976; Campbell and Peberdy, 1979). Chitin synthase preparation from *P. articulosus* and *C. cucurbitarum* were also activated by treatment with exogenous proteases (Table 1). The expressed activity and total activity was higher in *P. articulosus* preparation than those of *C. cucurbitarum*. However, the enzyme in the two species was activated by almost the same factor. Enzyme preparation from the two species responded differently to the treatment with various proteases. Acid protease was the most effective activator of zymogenic enzyme in the two fungi. Trypsin was comparatively less effective and neutral protease was inhibitory to *C. cucurbitarum* (Fig. 3). Similar results have been reported by Ruiz-Herrera *et al.* (1976), indicating that chitin synthase from *M. rouxii* can be activated best by acid protease and that from *Rhizopus* by Rennilase (Bartniki-Garcia, 1976), which on the opposite, was almost without effect on *Agaricus bisporus* mycelia chitin synthase. Hanseler *et al.* (1983) reported that trypsin, the protease most widely used to stimulate chitin synthase, was effective only to a limited extent

with the mushroom enzyme. It would appear that there exist different types of chitin synthases that are subjected to control by quite specific proteases.

The discovery by Cabib and Keller (1971), that a mild proteolytic digestion of chitin synthase preparation from yeast resulted in an increase in enzyme activity and the later confirmation of mild proteolytic activation of chitin synthase from filamentous fungi opened ways to speculation on the regulation of chitin synthase activity by proteases. Various models have been proposed to explain the regulation of chitin synthase activity *in vivo* and *in vitro*, involving proteases in the activation of this enzyme. A heat stable cytoplasmic proteinaceous inhibitor of chitin synthase has been isolated in *S. cerevisiae* (Cabib *et al.*, 1974) and in *M. rouxii* (McMurrough and Bartnicki-Garcia, 1973). It is believed that the inhibitor inhibits the activating factor and may function as a regulatory factor to inactivate the vacuolar proteases that spills into the cytoplasm hence preventing random stimulation of enzyme. However, their results cannot be accepted at their face value until more is known about the interaction between chitin synthase inhibitor and chitin synthase *in vivo*.

Low temperature storage studies suggested higher levels of endogenous protease in crude enzyme preparation from *C. cucurbitarum* than that from *P. articulosus* (Fig. 12). An endogenous neutral protease from *A. nidulans* which caused activation of chitin synthase zymogen 14 times greater than that obtained from trypsin treatment has been identified by Campbell and

Peberdy (1979). It is possible therefore that endogenous proteases may play an important role in the regulation of chitin synthase *in vivo*. The difference observed in the activation of zymogenic to active enzyme by various proteases in different fungi suggested that there might exist different types of chitin synthase that are regulated by quite specific proteases. An inhibitory component of chitin synthase was isolated in *M. vinacea* (Peberdy and Moore, 1975) and *M. rouxii* (Lopez-Romero *et al.*, 1978) which unlike that from *S. cerevisiae*, does not inhibit protease action on the zymogen but inhibits active chitin synthase directly. The role of chitin synthase inhibitor in filamentous fungi is yet to be established, but it has been suggested that it is a component of the regulatory mechanism of chitin synthesis (Lopez-Romero *et al.*, 1978).

From the present study, no conclusion can be made from the general requirements of the enzyme, as both the species showed similar requirements for enzyme activity. However, the characterization of the enzyme as a zymogen, and its activation by GlcNAc, Mg^{+2} and the differences in endogenous protease content in the two species confirm previous observations and provides some explanation for the regulation of chitin synthesis in *P. articulosus* and *C. cucurbitarum*. It remains to be explained how the activation of total chitin synthase occurs at a specific site and time.

Conclusion and Suggestions:

Zymogenicity is a universal property of fungal chitin synthetase. There are, however, some differences in the response of zymogen to specific proteases. Differences observed in the initial ratio of zymogen to active enzyme and in the stability of zymogen, during isolation may not necessarily reflect actual *in vivo* differences in activation and stability of chitin synthase in the two fungi, but may result mostly from differences in the nature and quantity of endogenous proteases released during cell breakage. The stability of chitin synthase from *C. cucurbitarum* at 4°C was much lower than that from *P. articulosus* enzyme preparations. In the former case, enzyme activity was lost after 5 h at 4°C, whereas the activity of the enzyme from *P. articulosus* decreased gradually with time, and shows significant activity after 24-30 hours' storage at 4°C. Since protease measurements indicate a slightly more endogenous proteases in *C. cucurbitarum*, incubation at 4°C might have resulted in a rapid degradation of the enzyme by endogenous proteolysis. The rather gradual decrease in enzyme activity observed in *P. articulosus* on storage could be due to low proteolytic activity or the presence of an inhibitory component tightly bound to the *P. articulosus* enzyme. This factor might have been lost during fractionation of *C. cucurbitarum* cells or might have been digested by the relatively higher levels of proteases observed in its crude extracts. This is further confirmed by results from endogenous protease studies using casein as substrate. Higher activities were found in both mixed membrane fraction (MMF) and supernatant fraction of *C. cucurbitarum*.

The exact physiological role of inactive chitin synthase in filamentous fungi is not well understood. In the present study there was no significant difference in the amount of inactive or zymogenic enzyme of the two species (Table 4). There was, however, a significant difference in total enzyme activity (Table 5), which leads one to speculate that total enzyme activity might be an important factor to consider in the resistance process. In the resistant host species, there might be localized hydrolytic activity which activates the host cell wall synthesizing enzyme. Whereas in the susceptible host, infection might have resulted in the suppression of the host's hydrolytic enzyme activity, hence suppression of host inactive cell wall synthesizing enzyme (chitin synthase). This probably results in the delay of host response to deposit chitinous cell wall-like material. It appears therefore that the full potential of chitin synthase activity is realized in a resistant host but not in a susceptible host. The mechanism of susceptibility seems therefore to depend on the specific suppression of localized hydrolytic activity which in turn regulates chitin synthase activity. The role of the parasite, if any, in localized hydrolytic activity or its suppression needs to be investigated.

Further studies should also be directed towards the role of endogenous proteases and inhibitors on purified chitin synthase preparations of the two species. In vivo studies should also be aimed at stimulating or inhibiting the endogenous proteases action, to find out the effect of this treatment on enzyme (chitin synthase) activity. In the present study

there was no direct evidence to confirm the assumption made in the *in vivo* study that host resistance during infection was due to activation of high levels of inactive enzyme. Results from the suggested study could throw more light on the regulation of chitin synthase activity in the two systems during infection.

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Appendix

Table A. Properties of some chitin synthase preparations from fungi.

Organism	Cell wall category*	'K_m' or $[S]_{0.5}$ for UDP-GlcNAc (mmol L^{-1})	Optimum pH	Optimum temperature °C	Optimum $[Mg^{+2}]$ (mmol L^{-})	Reference
Chytridiomycetes						
Blastocladiella emersonii	V. chitin-glucan	1.8-4.1	8	20-25	6-20	Camargo *et al.* (1967)
Zygomycetes						
C. cucurbitarum	IV. chitin-chitosan	1.7	6.2	23-25	10	unpublished
P. articulosus	IV. chitin-chitosan	2.5	6.2	25	10	unpublished
Zygomycetes						
Mucor rouxii	IV. chitin-chitosan	0.5-1.5	6.5	24-27	30	McMurrough and Bartniki-Garcia (1971)
Phycomyces blakesleeanus	IV. chitin-chitosan	0.6	6.5	28	20	Jan (1974)
Mortierella vinacea	IV. chitin-chitosan	1.8	6	32	20	Peberdy and Moore (1975)
Ascomycetes						
Saccharomyces cerevisiae	VI. mannan-glucan	0.6-0.9	6.2	37	10	Keller and Cabib (1971).
Neurospora crassa	V. chitin-glucan	1-2	7.5	27	--	Glaser and Brown (1957)
Basidiomycetes						
Coprinus cinereus	V. chitin-glucan	0.9	8	30	30	Gooday and de Rousset-Hall (1975)

* From Bartnicki-Garcia (1970)

Table 2. Properties of chitin synthase preparations (MMF) from mucoraceous host species, P. articulosus and C. cucurbitarum.

Organism	'K_m' for UDP-GlcNAc (mmol L^{-1})	Optimum pH	Optimum temperature °C	Optimum [GlcNAc] (mmol L^{-1})	Optimum [Mg^{+2}] (mmol L^{-1})	% inhibition by Polyoxin D (10 µg mL^{-1})
Choanephora cucurbitarum	1.7	6.2	23-25	20	10	94
Phascolymyces articulosus	2.5	6.2	25	20-30	10	94

Chitin synthase activity was assayed under different conditions as described in methods.

Table 3. Endogenous protease activity using two substrates:
(i) casein
(ii) Nα-benzoyl-DL-arginine-p-nitroanilide

Substrate	Protease activity (TU^{cas} μg^{-1} trypsin)			
	P. articulosus		C. cucurbitarum	
	MMF	SUP	MMF	SUP
Casein	20	5	25	6
Nα-benzoyl-DL-arginine-p-nitroanilide	--	--	--	--

(--) no measurable activity.